

# Direct analysis of artemisinin from *Artemisia annua* L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector

Congyue A. Peng<sup>a</sup>, Jorge F.S. Ferreira<sup>b,\*</sup>, Andrew J. Wood<sup>a</sup>

<sup>a</sup> Department of Plant Biology, Southern Illinois University-Carbondale, Carbondale, IL 62901-6509, USA

<sup>b</sup> USDA, ARS, Appalachian Farming Systems Research Center, 1224 Airport Rd., Beaver, WV 25813, USA

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## Abstract

Since the isolation of artemisinin 32 years ago, it has been analyzed by different chromatographic techniques. This work compared the analysis of artemisinin from crude plant samples by GC with flame ionization detection (GC–FID) and HPLC with evaporative light scattering detector (HPLC–ELSD). Data is also presented indicating that GC is suitable for the quantification of two of artemisinin precursors (arteannuin B and artemisinic acid) if a mass spectrometer is available. GC–FID and HPLC–ELSD were chosen because of their low cost compared to other detection methods, their ease of operation compared to HPLC with electrochemical detection, and because neither require artemisinin derivatization. Both GC–FID and HPLC–ELSD provided sensitive (ng level) and reproducible results for the analysis of artemisinin from field plants, with a correlation coefficient of  $r^2 = 0.86$  between the two methods. Both methods could be easily adapted to the analysis of pharmaceutical-grade artemisinin.

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**Keywords:** *Artemisia annua*; Artemisinin; Chromatography; HPLC; GC

## 1. Introduction

Artemisinin is a sesquiterpene lactone with a peroxide bridge, which is the effective moiety against both sensitive and multi-drug resistant *Plasmodium falciparum*, the malarial agent. Besides malaria, one of the artemisinin derivatives, dihydroartemisinin, was reported as effective in killing human breast cancer cell line HTB27 after incubation with holotransferrin [1]. Artemisinin has been quantified in the past by various analytical methods such as thin layer chromatography (TLC) [2,3], high-performance liquid chromatography with UV detection (HPLC–UV) after derivatization to a compound which absorbs at 260 nm [4], HPLC with electrochemical detection (HPLC–ECD) [5–7], HPLC with evaporative light scattering detection (HPLC–ELSD) [8–10], gas chromatogra-

phy with mass spectrometric detection (GC–MS) [11], GC with flame ionization detection (GC–FID) [6,12], and enzyme-linked immunosorbant assay (ELISA) [13]. Mass spectrometry has high sensitivity compared with other detectors, and the benefit of molecule confirmation through its major ions, but requires great investment and expertise. Detection of artemisinin by UV is the most affordable, but artemisinin must be derivatized due to the reported lack of chromophores in artemisinin [4]. Detection by GC–FID had problems in the past because one of artemisinin degradation peaks overlapped with arteannuin B, an artemisinin precursor [6]. Detection by HPLC–ECD is sensitive, specific, but oxygen must be removed from the mobile phase by continuously purging with Helium or Argon. Even when oxygen has been eliminated, it takes an EC detector over 1 h to stabilize before any injection can be made, and HPLC–ECD is also unable to detect artemisinin precursors (e.g., artemisinic acid, arteannuin B), which lack a peroxide group. This work presents the simultaneous analysis of artemisinin from plant samples by GC–FID and HPLC–ELSD without sample derivatization.

\* Corresponding author. Tel.: +1 304 256 2827; fax: +1 304 256 2921.  
E-mail address: [jorge.ferreira@ars.usda.gov](mailto:jorge.ferreira@ars.usda.gov) (J.F.S. Ferreira).

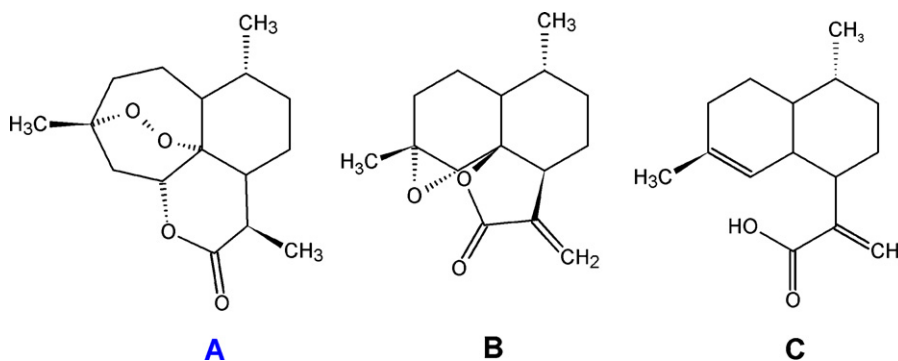


Fig. 1. Structures of artemisinin (A), and two of its bioprecursors arteannuin B (B), and artemisinic acid (C).

## 2. Experimental

### 2.1. Plant material and growth conditions

*Artemisia annua* seeds from the cultivar 3 M were donated by Dr. Pedro Magalhães from the CPQBA, Campinas, Brazil. Seeds were planted in trays in a greenhouse under a 16-h photoperiod under high-pressure sodium lamps (400 w). When plants were approximately 3.0 cm in height, they were transplanted into 6-in. pots. After screening for artemisinin content, 16 plants were vegetatively propagated under mist in a sand bed, and kept under a 16-h photoperiod until they were transferred to the field (Carbondale, IL, 37.73N, 89.209W) in June of 2003 when they had approximately 15 cm in height.

### 2.2. Standards

Artemisinin standards were purchased from Sigma (Saint Louis, MO, USA). Arteannuin B, artemisinic acid, artemisitene, and deoxyartemisinin were provided by Drs. Hala El-Sohly and Michael Avery (Ole Miss, University, MS).

### 2.3. Artemisinin extraction

Sixteen seed-generated lines of *A. annua* were harvested every 2 weeks after the plants were 3-month old. Harvest dates were September 13, October 1, October 17, and October 30 of 2003. At the last collection date, plants were in the flowering stage. For comparisons of the artemisinin concentrations determined by HPLC–ELSD and GC–FID, plants sampled on September 13 (middle and upper branches) were placed in a forced-air oven set at 50 °C for 48 h. Dried samples were sieved through a No. 14 mesh stainless steel sieve, and stored in stoppered glass jars at room temperature before artemisinin analysis. Samples were extracted and analyzed within 1 week of being harvested. Artemisinin extraction was performed by refluxing 0.5 g of sieved dry leaves with 50 ml of hexane at 75 °C for 1 h (>95% artemisinin recovery), following a modified procedure [6]. The hexane extracts were transferred to glass beakers and allowed to evaporate to dryness in a fume hood. Within 24 h, samples were reconstituted in 10.0 ml of acetonitrile, filtered through pre-wetted 0.2 µm (25 mm) nylon Millex-GN filters (Millipore Corporation, Bedford, MA) attached to disposable

3-ml syringes. This 10-ml extraction recovered 95–98% of the artemisinin extracted from the dry leaves by the hexane refluxing. Hexane refluxing was also effective in extracting arteannuin B and artemisinic acid (Fig. 1) from glandular trichomes. Filtered aliquots from the samples were transferred to HPLC and GC flasks and analyzed the same day.

### 2.4. HPLC–ELSD

HPLC analysis was performed by a Hitachi (Hitachi Technologies, Atlanta, GA) composed of a L-7100 gradient quaternary pump equipped with a degasser, a L-7250 programmable auto sampler, and a PL-ELS1000 ELSD (Polymer Laboratories, Amherst, MA), with the data collection through the Hitachi D-7000 HPLC System Manager software. The conditions on the ELSD were set as follows: evaporative temperature of 80 °C, nebulizer at 75 °C, and nitrogen flow at 0.8 L/min. The HPLC column was an endcapped Purosphere (Hitachi Technologies, Atlanta, GA) C18-RP 250 mm × 4.0 mm ID (5.0 µm pore size), kept at room temperature. The mobile phase was isocratic and constituted of water, adjusted to pH 3.0–3.5 with trifluoro acetic acid (TFA):acetonitrile (65:35) at the flow rate of 1.0 ml/min with a stop-time of 25 min.

### 2.5. GC–FID

Analysis of artemisinin and precursors was performed in a gas chromatographer GC-2010 (Shimadzu, Columbia, MD) equipped with a flame ionization detector, with the data collection through the Shimadzu GC-Solution software. The GC was set at the following conditions with helium as the carrier gas: pressure: 15.6 psi, total flow: 37.4 mL/min, column flow: 1.50 mL/min, linear velocity: 50.5 cm/s, purge flow: 3.0 mL/min, and a split ratio of 21:9. The column was a Rtx-5 crossbond 100% dimethyl polysiloxane (Resteck Corp), (15 m × 0.25 mm ID, 0.25 µm film thickness). Column temperature was set at 195 °C, injector at 240 °C, and FID temperature set at 300 °C, and sampling rate of 40 ms.

## 3. Results and discussion

Standards of artemisinin and its precursors arteannuin B and artemisinic acid were all used to calibrate both HPLC–ELSD

and GC–FID and to test instrument linearity and accuracy. Standards at concentrations of 0.03, 0.06, 0.125, 0.25, 0.5, and 1 mg/ml were freshly prepared and used in both instruments. Correlation coefficient ( $r^2$ ) in a linear plot was  $r^2 = 0.9727$  for the HPLC–ELSD standard curve, and  $r^2 = 0.9989$  for GC–FID standard curve. The better fit obtained with the GC–FID than with the HPLC–ELSD can be explained by the fact that the ELSD is not a linear detector. Although the linear regression between the two methods was done through linear fits, a better  $r^2$  can be obtained for the ELSD using a quadratic fit for the standard curve. Regarding precision and accuracy, a standard of 1.0 mg/ml of artemisinin was injected (10  $\mu$ l for the HPLC and 1  $\mu$ l for the GC) five times consecutively in each instrument. The intraday RSD (relative standard deviation) for GC–FID was 3.91%, and for the HPLC–ELSD was 1.4%. Both methods were under the 5–10% RSD accepted for chromatographic analysis of complex samples [14]. It is known that GC–FID degrades artemisinin and that artemisinin is measured through its major degradation peaks [6,10]. Under the instrument conditions used for this GC–FID method, standards of artemisinin and artemisitene were degraded (data not shown) to at least four breakdown products (peaks), while minor degradation occurred for artemisinin precursors. Under these conditions, artemisinin quantitation through its major breakdown product represented the true artemisinin content of the samples, previously determined by HPLC–ELSD (Figs. 2 and 3). Although previous analysis by GC–FID resulted in overlapping peaks of arteannuin B and one of the artemisinin degradation peaks [6], there was no overlapping when a Rtx-5 crossbond column was used instead of a DB-5 column used in a previous study [6]. Under the same conditions, a DB-5 column still generates overlapping peaks between arteannuin B and artemisinin (Ron Skinner, P.E.I. Food Technology Centre, Canada, personal communication). The retention time (rt) and peak area ratio for GC–FID of individual standards (not shown) at 1.0 mg/ml were as follows: artemisinic acid (rt=2.39, 100%), deoxyartemisinin (rt=4.35, 100%), arteannuin B with two peaks (rt=4.2, 0.8%; rt=4.65, 99.18%), artemisinin with four peaks (rt=3.4, 10.9%; rt=4.98, 14.6%; rt=6.95, 5.3%; rt=7.66, 55.4%). Artemisinin was estimated through the fourth peak, which represented 55.4% of the peaks, and which had the same molecular weight ( $m + 1 = 283$ ) as artemisinin (Ron Skinner, personal communication). This indicates that ca. 50% of the artemisinin is still intact at the temperatures the sample was submitted to during this GC–FID method. For artemisitene, four major peaks were obtained as follows: rt=3.63, 19.8%; rt=3.86, 22.6%; rt=5.69, 9.54%; rt=8.37, 41.23%, but no artemisitene was detected from the plant samples. Fig. 2C shows peaks generated by a standard mix of artemisinic acid (one peak with rt=2.39), arteannuin B (major peak with rt=4.65), and artemisinin (major peak with rt=7.66). Fig. 2D illustrates that only artemisinin was clearly detected from plant samples with GC–FID.

### 3.1. Artemisinin from field plants by HPLC–ELSD

A standard mix containing arteannuin B (0.3 mg/ml), artemisinin (0.3 mg/ml), and artemisinic acid (0.67 mg/ml) gen-

erated peaks at 6.07 min, 7.88 min, and 18.99 min, respectively (Fig. 2A). When plant samples were analyzed by HPLC–ELSD, artemisinin eluted at 7.63 min, and no artemisinic acid or arteannuin B could be detected in any of the 16 Brazilian (3 M, CPQBA) *Artemisia annua* plants (Fig. 2B). The absence of artemisinic acid can be expected due to the high temperatures of the evaporator (80 °C) and nebulizer (75 °C) used for this HPLC–ELSD method. Artemisinic acid was detected from standards at 0.05 mg/ml using a Polymerlab ELS 2000 with evaporative temperature of 40 °C and nebulizer at 30 °C, but not at the evaporative temperature of 70 °C (unpublished). Artemisinic acid and dihydroartemisinic acid levels are very low in plant samples, compared to artemisinin, and are better quantified through ultra-violet detection at 210 nm (Ferreira, unpublished). The second major peak present in plant samples, and eluting at 4.45 min, did not match the retention times of arteannuin B, artemisitene, deoxyartemisinin, or dihydroartemisinin from our compound library.

### 3.2. Artemisinin from field plants by GC–FID

The major peak (4th) generated by plant samples when analyzed for artemisinin by GC–FID eluted at 7.57 min (identity confirmation by GC–MS). Other peaks coinciding with the retention times of artemisinic acid, deoxyartemisinin, and arteannuin B eluted at 2.35, 4.3 and at 4.59 min, respectively (Fig. 2D). However, because a mass spectrometry was not available, and because the peaks were less than three-times larger than the baseline noise, we chose not to label those peaks as artemisinic acid, deoxyartemisinin, and arteannuin B. Several other peaks were present, but did not match any of the artemisinin compounds in our library. Thus, for these 16 *A. annua* plants, artemisinin was quantified through its major peak in the chromatogram generated by GC–FID, which ion matched artemisinin (Ron Skinner, personal communication). Although different chemical profiles were seen from plant to plant and from different collection dates for the same plant, for the GC–FID analysis of one of the seed-originated plants (GO), which contained 0.8% artemisinin on 9/13/03, artemisinin (rt=7.57 min) represented approximately 32% of the area of all peaks, while other major peaks (unidentified), before artemisinin, had the following representation in the chromatogram, according to their retention time: rt=3.4 (7.7%), rt=4.97 (2.9%), and rt=6.95 (52.0%). However, when the GO plant was collected on 10/17/03, artemisinin represented only 18.45% of the peak areas, while other major peaks, before artemisinin, had the following representation: rt=2.19 (21.6%), rt=3.07 (18.6%), rt=3.4 (10.4%), rt=4.3 (10.6%), rt=4.97 (11.1%), rt=5.4 (9.2%). This indicates that the chemical profile of *A. annua* changes during the season. From the first collection time (9/13/03), artemisinin content in all 16 field-grown plants decreased consistently with later collection dates. This increased artemisinin content observed in mid September coincided with the beginning of the flowering stage for that Brazilian cultivar, which is consistent with the artemisinin peak also observed in Campinas, Brazil, where the cultivar was developed (Pedro Melillo de Magalhães, personal communication).

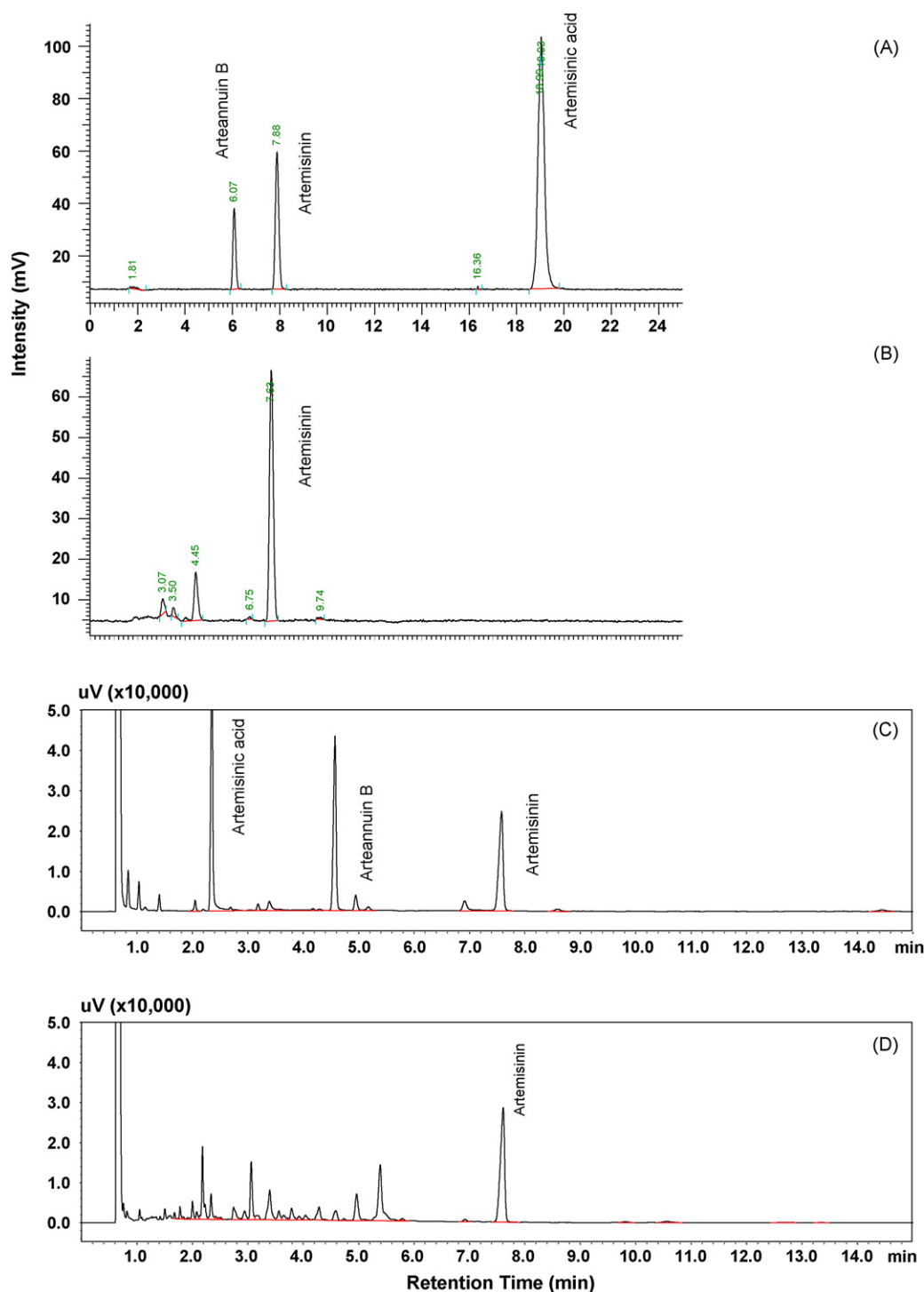


Fig. 2. Chromatograms of standards of artemisinin and its precursors (A) and plant extracts (B) by HPLC–ELSD, and standards of artemisinin and its precursors (C) and plant extracts (D) by GC–FID. (A) Chromatograms generated by HPLC–ELSD of standards of arteannuin B (rt=6.07), artemisinin (rt=7.88), artemisinic acid (rt=18.99). (B) Chromatograms generated by HPLC–ELSD of extracts of *A. annua* (3 M, CPQBA) dry leaves (50 mg/ml), artemisinin rt=7.63 B). (C) Chromatograms generated by GC–FID of standards of artemisinic acid (rt=2.37), arteannuin B (rt=4.60), and artemisinin (rt=7.56) analyzed by GC–FID. (D) Chromatograms generated by GC–FID of extracts (50 mg/ml) of *A. annua*; artemisinin rt=7.61. Standard concentrations for arteannuin B and artemisinin were 0.3 mg/ml, and 0.67 mg/ml for artemisinic acid.

Artemisinin identification and quantification was made by comparing its retention time of the peak area found in plant samples with the artemisinin peak from a standard mix injected with each batch of samples. For the GC–FID analysis, artemisinin identification and quantifica-

tion from plant samples was performed through its major degradation peak, compared to a standard mix (Fig. 2C). The separation through Rtx-5 dimethyl polysiloxane column reveals the major artemisinin degradation peak at rt=7.56. (Fig. 2D).

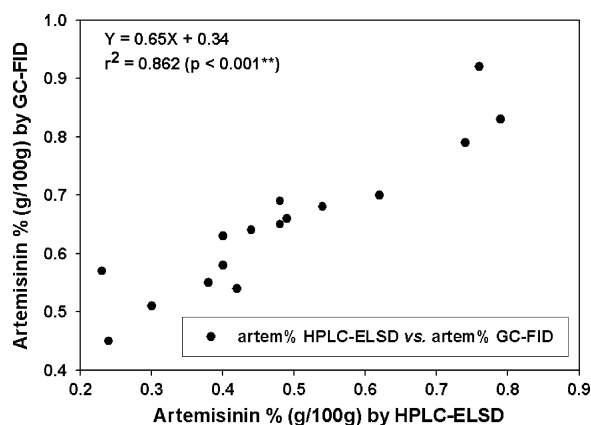


Fig. 3. Correlation of artemisinin content (w/w %) of 16 seed-generated lines of *A. annua* (3 M, CPQBA) analyzed by both HPLC–ELSD and GC–FID. These plants were field grown in Illinois and harvested on September 13, before flowering. Their artemisinin content varied from 0.226% to 0.785% (w/w).

In order to validate both HPLC–ELSD and GC–FID methods, the 16 seed-generated lines of *A. annua*, ranging from 0.2% to close to 0.9% from the 9/13/03 collection date, were extracted and analyzed for their artemisinin content by both GC–FID and HPLC–ELSD. Artemisinin content was calculated from calibration curves generated by the known concentration of artemisinin standards, through their peak area. A significant correlation ( $r^2 = 0.862$ ,  $p < 0.001^{**}$ ) was obtained when the concentration (g/100 g) of artemisinin from the 16 clones was compared by HPLC–ELSD and GC–FID (Fig. 3). The results obtained for GC–FID agreed with the ones obtained by HPLC–ELSD, in which artemisinin was the major identified peak detected from plant samples.

We conclude that both methods provide fast and reliable determination of artemisinin from plant samples even when artemisinin decreased to a minimum during the plant life cycle (data not shown), but we could not confirm the identity of artemisinin precursors without mass spectrometric detection. The limit of detection (LOD) of HPLC–ELSD, based on the ng on column was around 50 ng for artemisinin, 100 ng for arteannuin B, and over 500 ng for artemisinic acid. Thus, artemisinic

acid should not be quantified by HPLC–ELSD. LOD established for the GC–FID, based on ng on column (with standards) was around 30 ng for artemisinin, 4 ng for artemisinic acid, and 5 ng for arteannuin B. One must consider that HPLC–ELSD, analyzes artemisinin as a whole molecule while GC–FID analyzes artemisinin through its major degradation products, but also that a GC–FID system costs about half of the price of the HPLC–ELSD system.

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